Optimization of biomethanation focusing on high ammonia loaded processes

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Preface

This PhD thesis, entitled "Optimization of biomethanation focusing on high ammonia loaded processes", comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from November 01, 2012 to February 29, 2016. Professor Irini Angelidaki and researcher Ioannis Fotidis were supervisor and co-supervisor, respectively.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred in the text by their paper number, which are written with the Roman numerals **I-IV**.

I Fotidis, I.A., Wang, H., Fiedel, N.R., Luo, G., Karakashev, D.B., Angelidaki, I. 2014. Bioaugmentation as a solution to increase methane production from an ammonia-rich substrate. *Environmental Science & Technology*, 48(13), 7669-7676. DOI: 10.1021/es5017075.

II Wang, H., Fotidis, I.A., Angelidaki, I. 2015. Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate-oxidizing bacteria. *FEMS Microbiology Ecology*, 91(11), fiv130. DOI: http://dx.doi.org/10.1093/femsec/fiv130.

III Wang, H., Fotidis, I.A., Angelidaki, I. 2016. Ammonia - LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process. *Bioresource Technology*. Accepted.

IV Wang, H., Zhang, Y., Angelidaki, I. 2016. Ammonia effect on hydrogen assisted biogas production and upgrading process. Submitted.

In this online version of the thesis, papers **I-IV** are not included but can be obtained from electronic article databases, e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

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Summary

The toxicity effect of high ammonia is one of the most common problems, which cause imbalance and low biogas production rate in biogas plants. When protein-rich substrates (e.g. pig manure and mink manure, food waste, etc.) are used in biogas plants, lead to suboptimal utilization of the biogas potential and unstable biogas process. However, up to now, the solutions for alleviating ammonia toxicity effect have been proven either too expensive or time consuming for the full-scale biogas plants. Thus, sustainable and practical solutions to overcome the problem of ammonia inhibition efficiently are urgently required. In order to alleviate the toxicity effect of high ammonia levels, some new ideas-hypotheses were presented and evaluated in this thesis.

Firstly, preliminary modelling results from a previous study, have demonstrated that the increase of lipids' concentration in ammonia-rich substrates, could theoretically mitigate the ammonia inhibition problem (Angelidaki et al., 1999). Therefore, the effect of co-digestion of cattle manure with lipids (i.e. glycerol trioleate (GTO)) under high ammonia levels (5 g NH_4^+-N·L^{-1}) in anaerobic continuous stirred tank (CSTR) reactors (R_{GTO}) was assessed. Additionally, for comparison purposes, a soluble carbohydrate (i.e. glucose) was also used as a co-substrate in an identical CSTR reactor (R_{GLU}). At 5 g NH_4^+-N·L^{-1}, relative methane production of R_{GTO} and R_{GLU}, was 10.5% and 41% compared to the expected uninhibited production, respectively. At the same time control reactor (R_{CTL}), only fed with manure, reached 32.7% compared to the uninhibited basis production. Therefore, the hypothesis that the co-digestion of manure with lipids could alleviate the ammonia inhibition was not supported by the results. However, an "ammonia-LCFA synergetic inhibitory effect" was observed, which caused a deterioration of the inhibition effect in anaerobic digestion process. On contrary, the reactor where glucose was co-digested demonstrated higher tolerance to ammonia toxicity compared with the reactor where GTO was used.

Secondly, the problem of ammonia inhibition during biomethanation process could also be solved by microbiological methods. It is possible to promote the syntrophic acetate oxidation pathway during biomethanation process for counteracting ammonia inhibition. Therefore, the effects of different ammonia levels on pure strains of syntrophic acetate oxidation bacteria (SAOB) and hydrogenotrophic methanogens were evaluated. Furthermore, the effect of different ammonia levels on the syntrophic cultivation of SAOB and hy-

drogenotrophic methanogens was also assessed. The results showed that some hydrogenotrophic methanogens (79.1% of the theoretical methane production) were equally, or more resistant to ammonia toxicity compared to SAOB (11.1% of the theoretical methane production). In addition, the thermophilic hydrogenotrophic methanogens tested in the current study were more robust to high ammonia concentrations compared to the mesophilic hydrogenotrophic methanogens, which was contradictory to the results of some previous studies. Moreover, for SAOB, the resistance to ammonia toxicity could be improved by syntrophic cultivation with hydrogenotrophic methanogens, which indicated that at high ammonia levels, hydrogenotrophic methanogens seem to be the key players in the SAO pathway.

Thirdly, based on the same idea (promoting the syntrophic acetate oxidation pathway to alleviate ammonia inhibition), the hypothesis of bioaugmentation with high ammonia tolerant methanogenic archaea could be a new practical solution for fast recovery from ammonia inhibition. The results derived from this study clearly demonstrated a 31.3% increase in methane production yield in the CSTR reactor, at steady-state, after bioaugmentation. It indicated that this new solution to counteract ammonia inhibition was more practical and effective compared with other methods applied today in continuous reactors. Furthermore, bioaugmentation with an ammonia tolerant methanogen to alleviate ammonia toxicity could be applied for improving the efficiency of biomethanation process in full-scale continuous reactors.

Finally, an innovative method, where hydrogen is injected in the anaerobic reactor and subsequently been converted together with carbon dioxide to methane by hydrogenotrophic methanogens, could potentially be more tolerant to ammonia toxicity. Therefore, the effect of different ammonia levels on this hydrogen assisted biogas upgrading process under different hydrogen partial pressure (0, 0.25, 0.5 and 1 atm) in anaerobic reactors at both mesophilic and thermophilic temperature was evaluated. When the initial hydrogen partial pressure was 0.5 atm, the methane yield at high ammonia load (7 g NH_4^+-N L^{-1}) was 41.0% and 22.3% lower than at low ammonia load (1 g NH_4^+-N L^{-1}) in mesophilic and thermophilic condition, respectively. For the reactors without adding hydrogen, the methane yield decreased 65.0% (mesophilic) and 44.2% (thermophilic) when ammonia level increased to 7 g NH_4^+-N L^{-1}. The results demonstrated that the hydrogen assisted biogas production and upgrading processes were inhibited by high ammonia levels. Nevertheless, the hydrogen assisted biogas upgrading process was still more robust to the increasing ammonia concentrations compared to the conventional anaerobic

digestion processes. Under all the different ammonia concentrations tested in the current study, the optimal hydrogen partial pressure in batch reactors was 0.5 atm. Furthermore, at 0.5 atm of hydrogen partial pressure, the thermophilic methanogens seemed to be more robust to high ammonia concentrations (5 and 7 g NH_4^+-N L^{-1}) compared with mesophilic methanogens.

Dansk sammenfatning

Den toksiske effekt af højt ammoniakniveau udgør et af de mest almindelige problemer i biogasanlæg, som forårsager proces ubalance og nedsat produktion af biogas. Brug af proteinrige substrater (fx gødning fra grise, madaffald, etc.) i biogasanlæg fører til suboptimal udnyttelse af biogaspotentialet og en ustabil biogasproces. Samtidig har det vist sig, at de hidtidige løsninger til at dæmpe den toksiske effekt af ammoniak i fulskala biogasanlæg enten har været for dyre eller tidskrævende. Dermed er der et presserende behov for udvikling af bæredygtige og praktisk mulige løsninger til effektivt at overvinde problemerne med hæmning af biogasproduktion forårsaget af ammoniak. Denne PhD-afhandling præsenterer og evaluerer nye idéer og hypoteser til at dæmpe den toksiske effekt af høje ammoniakniveauer i biogasanlæg.

For det første viste foreløbige modelleringsresultater fra et tidligere studie, at forøgelsen af koncentrationen af lipider i ammoniak-rige substater teoretisk set vil kunne nedsætte problemet med hæmning forårsaget af ammoniak (Angelidaki et al., 1999). Med afsæt i disse resultater, blev effekten af samudrådning af kvæg-gylle med fedtstoffer (glycerol trioleat (GTO)) under højt ammoniakniveau (5 g NH_4^+-N·L^{-1}) i anaerobe fuld-omrørt reaktor analyseret. Til sammenligning blev der yderligere anvendt en opløselig kulhydrat (glucose) som co-substrat i en identisk CSTR reaktor. Resultaterne understøttede ikke den bagvedliggende hypotese, at co-digestion af gødning med lipider kan dæmpe den toksiske effekt af ammoniak. Dog observeredes en "ammoniak-LCFA synergisk hæmmende effekt", som forårsagede en forstærket hæmning i den anaerobe udrådnings proces. Til gengæld udviste reaktoren, hvor glucose var co-digested, en højere tolerance overfor den toksiske effekt af ammoniak sammenlignet med reaktoren hvor GTO blev brugt.

For det andet kunne problemet med ammoniak hæmning af biomethanerings process også løses ved mikrobielle metoder. Det er muligt at promovere den syntrofe oxidering af acetat under biomethanerings processen og herved modvirke hæmningen fra ammoniak. Derfor blev effekten af forskellige ammoniakniveauer på rene kulturer af syntrofiske acetat oxiderende bacteria (SAOB) og hydrogenotrofe methanogener evalueret. Ydermere blev effekten af forskellige ammoniakniveauer på den syntrophiske kultivering af SAOB og hydrogenotrofe methanoger analyseret. Resultaterne viste, at nogle hydrogenotrofe methanogener var lige så eller mere resistente over for den toksiske effekt af ammoniak sammenlignet med SAOB. Derudover viste resultaterne, at de termofile hydrogenotrofe methanogener, som blev test of nærværende

PhD, var mere robuste overfor høje ammoniakkoncentrationer sammenlignet med mesophile hydrogenotrophiske methanogener, hvilket var i modstrid med tidligere undersøgelser. Ydermere blev det for SAOB fundet, at resistensen overfor den toksiske effekt af ammoniak kunne blive forbedret via syntrophisk kultivering med hydrogenotropiske methanogener. Sidstnævnte indikerede, at under høje ammoniakniveauer udgør hydrogenotrophiske methanoger væsentlig rolle i SAO pathway.

For det tredje, baseret på den samme idé (at promovere syntrofiske acetat oxiderende pathway for at dæmpe ammoniaks hæmmende effekt), kunne hypotesen om bioaugmentering med methaogene archea, som har en høj tolerance for ammoniak, blive en ny praktisk løsning for hurtig gendannelse efter ammoniakhæmning. Resultaterne fra denne PhD indikerede, at denne nye løsning til at modvirke ammoniakhæmning var mere praktisk og effektiv sammenlignet med andre metoder anvendt i dag i kontinuerte reaktorer. Ydermere kunne bioaugmentering med en ammoniaktolerant methanogen anvendes til at øge effektiviteten af biomethanation-processen i fuldskala kontinuerte reaktorer.

Endelig er det fundet at en innovativ metode, hvor hydrogen er injekteret i den anaerobiske reaktor og efterfølgende konverteret sammen med carbondioxid til methan faciliteret af hydrogenotrophiske methanogener, potentielt set kunne være mere tolerant overfor ammoniaks toksicitet. Derfor blev effekten af forskellige ammoniakniveauer på denne hydrogen-assisterede opgraderingsproces af biogas i anaerobe reaktorer evalueret. Evalueringen foregik under forskellige hydrogen partielle tryk (0, 0.25, 0.5 og 1 atm) og ved både mesophile og termophile temperaturer. Resultaterne demonstrerede at den hydrogenassisterede biogasproduktion og opgraderingsproces blev hæmmet af høje ammoniakniveauer. Ikke desto mindre var den hydrogenassisterede opgraderingsproces af biogas stadigvæk mere robust overfor stigende ammoniakkoncentrationer sammenlignet med den konventionelle anaerobiske digestion proces. For alle de forskellige ammoniakkoncentrationer, som blev testet i denne PhD, var et hydrogen partielt tryk på 0.5 atm det optimale tryk. Ved 0.5 atm hydrogen partielt tryk, så de termophile methanogener ud til at være mere robuste overfor høje ammoniakkoncentrationer (5 og 7 g NH_4^+-N L^{-1}) sammenlignet med mesophile methanogener.

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1 Introduction

1.1 Background

Biogas production is usually obtained from waste treatment. The substrates for biogas production are mostly sewage sludge, agricultural waste (manure), and industrial waste (wastewater) (Hartmann & Ahring, 2005). Among all the feedstocks, the manure, mostly from cows or pig farms, is considered as the main substrate, which could supply all the necessary microorganisms for degrading the biomass and is one of the largest single biomass sources from the food industry. In the EU-27 (27 countries in European commission), there is a rich production of manure: more than 1,500 million tons is produced every year (Nielsen et al., 2007b). Table 1 shows the biogas and energy potential of cattle and pig manure in the EU-27.

Table 1. Energy potential of pig and cattle manure in EU-27

Total manure, Mt[a]	Biogas, Mm^3	Methane, Mm^3	Potential, PJ	Potential, Mtoe
1,578	31,568	20,519	827	18.5

[a]Mt (million tons), Mm^3 (million cubic meter); Mtoe (million tons oil equivalent); 1 Mtoe = 44.8 PJ; Methane heat of combustion: 40.3 MJ/m^3; Assumed methane content in biogas: 65%.

In Denmark, about 30 million tons of manure is produced every year. However, only 5-7% of them are used to produce biogas. Therefore, a large potential of biogas production, which stored in the left unused manure is waiting for the exploiting in a sustainable way in the future.

By the year 2020, 50% of the manure would be used to produce biogas in a plan made by Danish government. Thus, there will be much more biogas plants being set up for accomplishing the plan and it is an irreversible tendency for using renewable energy instead of traditional energy in the future. However, there still are some problems in biogas plants.

The toxicity effect of high ammonia is one of the most common problems, which cause imbalance and low biogas production rate in biogas plants. When protein-rich substrates (e.g. pig manure and mink manure, food waste, etc.) are used in biogas plants, lead to suboptimal utilization of the biogas potential and unstable biogas process. The problem of ammonia inhibition has been known for years and free ammonia is considered as the main toxic factor. Therefore, anaerobic digestion process for producing biogas and the mechanisms of ammonia inhibition were introduced as followed.

1.2 Anaerobic digestion

Anaerobic digestion is a widely applied biotechnology process and has been evaluated as one of the most energy-efficient and environmentally beneficial technology for biogas production (Fehrenbach et al., 2008). Four steps are included in anaerobic digestion process: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Different groups of microorganisms play specific roles in different steps during the complex process (Angelidaki et al., 2011). The specific steps were shown in figure 1.

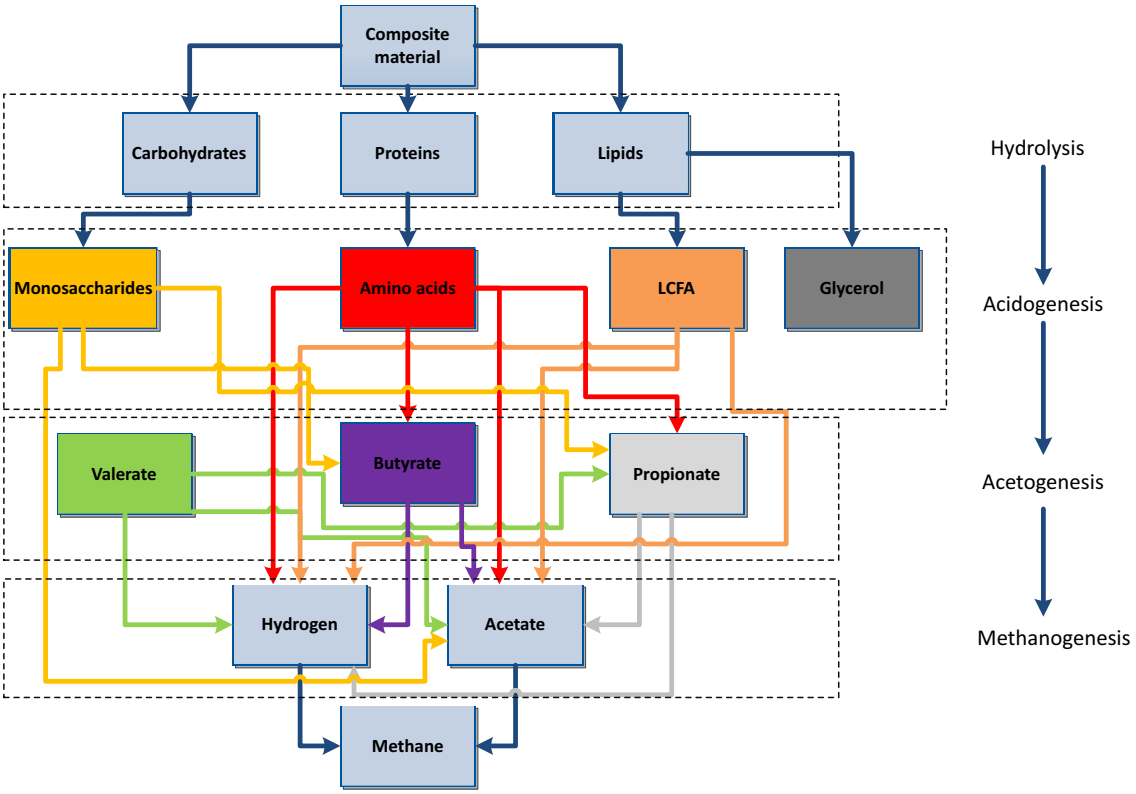

Figure 1. The specific four steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) during anaerobic digestion process.

1.2.1 Hydrolysis

Carbohydrates, proteins, and lipids are the three main organic compounds used as substrates during hydrolysis in which are converted to monosaccharides, amino acids, long chain fatty acids and glycerol, respectively. Specifically, the hydrolysis of each substrate was described as followed.

Firstly, biofibers (carbohydrates) are a mixture of cellulose, hemicellulose, and lignin (Tong et al., 1991). The structure and composition are the crucial factors for the degradation of biofibers. For example, lignin could not be de-

graded and materials with available structure (straw) can be hydrolysed (Tong et al., 1991; Yang et al., 2009). Secondly, the structure is also essential for the degradation of protein. In detail, the structure of fibrous proteins is more difficult to hydrolyse compared with semi soluble globular proteins (McInerney, 1988). Finally, for the degradation of lipids, different environmental conditions (surface tension and pH) and particle sizes rather than chemical structure caused different hydrolysis rates, which are different to the degradation of biofibers and protein.

In addition, in this step, hydrolysis rate is considered to be slow and widely regarded as the rate-limiting step of degradation of substrate during anaerobic digestion process (Pavlostathis & Giraldo-Gomez, 1991).

1.2.2 Acidogenesis

The products from the hydrolysis step (monosaccharides, amino acids, long chained fatty acids and glycerol) are converted into different fermentation products (valerate, butyrate, propionate, acetate, hydrogen, alcohols and acetate, etc.) during the step of acidogenesis. Monosaccharides and amino acids are two main substrates in acidogenesis step. Moreover, the pathways for fermenting the two main substrates are thoroughly different.

Emben–Meyerhof–Parnas (EMP) or Entner Doudoroff (ED) pathway is used for the fermentation of monosaccharides, which is a widely applied biotechnology process all over the world. In detail, the fermentation products are lactate and propionate (C3 products) through EMP pathway. Acetate, butyrate and caproate (C2/C4/C6 products) are produced through acetyl-CoA. In addition, ethanol, acetate and butyrate are considered to be the most common products from fermentation (Rodríguez et al., 2006).

The Stickland reaction is the metabolic pathway for fermentation of amino acids (Winter et al., 1987). The differences between the fermentation mechanisms of amino acids and monosaccharides are as followed: two amino acids are degraded as a couple in the oxidation/reduction reaction in which one amino acid is an electron acceptor, while the other is an electron donor. On the other hand, glucose plays both electron acceptor (e.g., acetate) and donor (e.g., propionate, ethanol, etc.) all by itself. However, there still exists the fermentation of uncoupled amino acid. For instance, the degradation of glutamate is an uncoupled fermentation (Buckel, 2001). Moreover, uncoupled amino acid oxidation could also happen when the energetics are appropriate and hydrogen pressures are low (Stams, 1994). For example, instead of pro-

ducing acetate from glycine during the fermentation of coupled amino acids, alanine uses electrons to generate two molecules of hydrogen.

In addition, during the process of fermentation, the degradation of amino acids will produce ammonia, which is a toxic compound for inhibiting the activities of microorganisms.

1.2.3 Acetogenesis

In the step of acetogenesis, acetate is produced from the fermentation products (propionate, butyrate and ethanol, etc.) by a) hydrogen-producing acetogens; or b) hydrogen-utilizing acetogens (Drake, 1994).

Specifically, organic acids (propionate and butyrate) are oxidized to generate acetate in which carbon dioxide and hydrogen ions are used as electron acceptor. Due to the unfavourable energetics of the acetogenesis reaction, the activities of hydrogen-producing acetogens are restricted. Acetate and hydrogen are the products of the acetogenesis and, at the same time, the substrates for the next step (methanogenesis). Therefore, hydrogenotrophic methanogens and sulfate reducers, which can consume hydrogen to keep its partial pressure low, are crucial for the acetogenesis process (McInerney et al., 2008; Sousa et al., 2009; Stams & Plugge, 2009). Thus, the interspecies hydrogen transfer is accomplished by the cooperation of hydrogen-producing acetogens and hydrogenotrophic methanogens.

The obligatory syntrophic association between hydrogen-producing acetogens and hydrogenotrophic methanogens has some unique characteristics:

- The degradation of propionate and butyrate has to be based on the cooperation of acetogens and methanogens.
- The specific growth rates are affected by the interspecies distances between hydrogen-producing acetogens and hydrogenotrophic methanogens (Batstone et al., 2006).
- The sharing of the available chemical energy during the syntrophic metabolism of the microorganisms is also a characteristic.

In addition, hydrogen-utilizing acetogens produce acetate by the reduction of carbon dioxide (the acetyl-CoA pathway) (Drake, 1994). These acetogenic bacteria are competing with the hydrogenotrophic methanogens for the available hydrogen, methanol and formate.

1.2.4 Methanogenesis

Methanogenesis is the final step of anaerobic digestion process where acetate, carbon dioxide with hydrogen primarily and formate, methyl and alcohols, secondarily are converted into methane by methanogenic archaea (Thauer et al., 2008). Acetate is the main substrate for producing methane; 70% of the total global methane is come from acetate, while the other 30% is generated from hydrogen and carbon dioxide, or formate (Conrad et al., 2010). There are two different pathways for consuming acetate: the aceticlastic pathway and the syntrophic acetate oxidation (SAO) pathway. Specifically, in aceticlastic pathway, methane and carbon dioxide are produced from acetate by aceticlastic methanogens (Angelidaki et al., 2011).

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^{0'} = -31.0\ kJ \cdot mol^{-1} \qquad (1)$$

In SAO pathway, there are two steps: first, hydrogen and carbon dioxide are produced from acetate by syntrophic acetate oxidation bacteria (SAOB). Second, hydrogen and carbon dioxide generated from the first step are used by hydrogenotrophic methanogens to produce methane (Zinder & Koch, 1984)

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \qquad \Delta G^{0'} = +104.6\ kJ\ mol^{-1} \qquad (2)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad \Delta G^{0'} = -135.6\ kJ \cdot mol^{-1} \qquad (3)$$

1.3 Anaerobic digestion microbiology

During anaerobic digestion process, different groups of microorganisms play specific roles in different steps. Specifically, during the steps of hydrolysis and acidogenesis, fermentative bacteria participate in secreting extracellular enzymes for degrading substrates (carbohydrates, proteins, and lipids) and in the fermentation of monosaccharides, amino acids and long chained fatty acids. For examples, hydrolytic bacterium *Cellulomonas* is able to hydrolyse cellulose by breaking the bonds between glucose. *Clostridium* and *Propionibacterium* participate in the degradation of monosaccharide, which producing butanol, butyrate and isopropanol, acetate and propionate, respectively.

During the step of acetogenesis, as it introduced above (Chapter 1.2.3), acetate is produced from fermentation products by the cooperation of hydrogen-producing acetogens (*Syntrophomonas wolfei*, *Thermobacteroides proteolyticus* and the syntroph PA-1, etc.) and hydrogenotrophic methanogens or formed from hydrogen and carbon dioxide by hydrogen-utilizing acetogens (*Acetoanaerobium romashkovii*, *Acetobacterium bakii* and *Acetoanaerobium noterae*, etc.) using the acetyl-CoA pathway.

In the final step of anaerobic digestion, several groups of microorganisms are involved, which are strictly anaerobic archaea. Specifically, as it introduced above (Chapter 1.2.4), aceticlastic methanogens (i.e. *Methanosaetaceae* spp. and *Methanosarcinaceae* spp.) are responsible for the aceticlastic pathway. On the other hand, SAOB and hydrogenotrophic methanogens (i.e. *Methanomicrobiales* spp., *Methanococcales* spp., *Methanocellales* spp., *Methanobacteriales* spp. and *Methanopyrales* spp.) combined to produce methane in SAO pathway.

In addition, temperature and pH growth range are the main physiological characteristics for methanogenic archaea. There is a wide range of temperature for different methanogenic archaea. In detail, for the majority of known methanogens, the suitable temperatures are from mesophilic (37°C) to thermophilic (55°C). However, there still exists *Methanopyrus kandleri*, a strict hyperthermophilic strain, which grows at 110°C (Kurr et al., 1991). Additionally, some previous studies showed that several methanogenic archaea (*Methanosarcina lacustris* and *Methanogenium frigidum*) grow at low temperature (Garcia et al., 2006; Kendall & Boone, 2006). Moreover, for the growth of most methanogenic archaea, the optimal pH level is around 7. However, *Methanosarcina baltica* (Kendall & Boone, 2006) and *Methanothermococcus okinawensis* (Whitman & Jeanthon, 2006) prefer growing at an environment of lower pH level (4-4.5) while *Methanosalsum zhilinae* (Kendall & Boone, 2006) and *Methanothermococcus thermolitotrophicus* (Whitman & Jeanthon, 2006) like alkaline environment (growing under pH of 9.8-10).

1.4 Ammonia inhibition on anaerobic digestion

During anaerobic digestion process, many inhibitors could cause inhibition (such as ammonia, sulfide, light metals, heavy metals and organics, etc.). Among these factors affecting the performance of the process, ammonia has been considered as the main factor that affects the stability of anaerobic digestion and then causes inhibition and suboptimal biogas production. Thus, ammonia inhibition was introduced as followed.

There is a delicate biochemical balance between the acidogenic microorganisms and methanogenic archaea, which keeps the stability of anaerobic digestion process. Ammonia, which is generated during the biological degradation of proteins, nucleic acids and urea (González-Fernández & García-Encina, 2009), can break the balance and results in restraining activities of microorganisms and an instability of the process (Liu & Sung, 2002; Zhang et al.,

2011). In all the different types of microorganisms involved in anaerobic digestion process, the growths of methanogens are the most sensitive and vulnerable to high ammonia levels (Kayhanian, 1994). Therefore, the unbalance between the acidogenic microorganisms and methanogenic archaea causes a decrease of methane production rates and an accumulation of the intermediate digestion products such as volatile fatty acids (VFA) (Calli et al., 2005; Sung & Liu, 2003).

The mechanisms of ammonia inhibition have been studied in some previous researches. There are two principal forms of inorganic ammonia nitrogen in aqueous solution: Ammonium ion (NH_4^+) and free ammonia (NH_3). Free ammonia is considered as the main toxic compound, which causes ammonia inhibition. In detail, Sprott and Patel (1986) and Gallert et al. (1998) reported that the passive diffusing of free ammonia molecule into the microbes cells results in proton imbalance, increase maintenance energy requirements, potassium deficiency and suppress specific enzyme reactions. In addition, there are several factors affecting the toxicity of ammonia inhibition, which were introduced as followed.

1.4.1 Ammonia concentration

There are many researchers that have studied the inhibition levels of ammonia and/or free ammonia on anaerobic digestion process. For example, a decrease of 39% in specific activity of methanogens in continuous stirred tank reactors was observed when the total ammonia levels were increased to 4.92 g NH_4^+-N L^{-1} (55°C, pH 6.71). Also in the same study, as total ammonia levels was higher than 4.0 g NH_4^+-N L^{-1} (55°C, pH from 6.5 to 8.0), a serious inhibition occurred on methanogens in batch experiments (Sung & Liu, 2003). The results of another study showed that when swine manure was co-digested with solid parts from manure at 1450 mg NH_3-N L^{-1} (51°C, pH >7.6) in continuous stirred tank reactors, there is a reduction of 50% for methane production (Nakakubo et al., 2008). Hansen et al. (1998) found the ammonia inhibitory effect at 750 mg NH_3-N L^{-1} (37°C, pH 8.0) with an increased VFA concentrations of 4800 mg/L. Overall, the wide range of ammonia concentrations causing inhibition in different studies can be explained by the differences in operational conditions (such as environmental conditions (temperature, pH), organic loading rate and substrates, etc.) (Angelidaki & Ahring, 1994; De Baere et al., 1984; Hashimoto, 1986; Van Velsen et al., 1979). Moreover, different types of reactors can cause different inhibiting ammonia levels. Specifically, in batch experiments, the decreasing of the methane production rates or growth rates are observed at the initial inhibition levels (Liu

et al., 2008). On the other hand, the inhibition could only be detected when the washout effect occurred in continuous reactor experiments.

1.4.2 Temperature

Temperature can affect anaerobic digestion process by changing the activities of microorganisms and free ammonia concentrations.

In detail, free ammonia concentration is affected by temperature, pH level and total ammonia concentration in anaerobic digestion process (Chen et al., 2008). Specifically, the conversion from NH_4^+ to NH_3 is improved when pH level and temperature are increased and causes an enhanced ammonia inhibition on the anaerobic digestion process (Angelidaki & Ahring, 1994). The free ammonia concentrations were calculated based on the following equation (Fotidis et al., 2013b):

$$\mathrm{FAN} = \frac{\mathrm{TAN}}{1 + \frac{10^{-\mathrm{pH}}}{K_a}}$$

Where TAN is total ammonia nitrogen, K_a is a dissociation constant that reflects on temperature with values 1.29×10^{-9} and 3.91×10^{-9} for 37 and 55 °C respectively.

Some researchers believed that anaerobic reactors could be more stable and robust to high ammonia levels in mesophilic condition compared with thermophilic condition due to the lower free ammonia concentration (Braun et al., 1981; Parkin & Miller, 1983). Angelidaki and Ahring (1994) reported that in anaerobic reactors with a high free ammonia level, an increasing of methane production yield and a relief of inhibition were observed when the operating temperature was reduced from 60 °C to 37 °C. However, contrary results were obtained by Gallert and Winter (1997). They found that with the temperature condition changed from mesophilic to thermophilic, the inhibiting free ammonia level increased from 220 to 690 mg NH_3-N L^{-1}, which indicated that thermophilic methanogens were more tolerated to free ammonia compared with mesophilic methanogens.

1.4.3 pH level

As it described above, pH level can also affect the toxicity of ammonia inhibition by changing free ammonia concentrations (Figure 2). For example, control of pH may alleviate the ammonia toxicity in anaerobic digestion. In Braun et al. (1981)'s study, liquid piggery manure was used as substrate for anaerobic digestion and when pH level was reduced from 8 to 7.4, the accu-

mulated VFA concentration decreased from 316 mg/L to 20 mg/L. The relief of the ammonia inhibition could be explained by the lower free ammonia concentration in the reactor at the reduced pH level (7.4). Moreover, another study indicated that keeping the pH level within the optimal range is essential for the growth of microorganisms. The anaerobic digestion process could still fail at an inappropriate pH level although the ammonia concentration is not high (Kroeker et al., 1979).

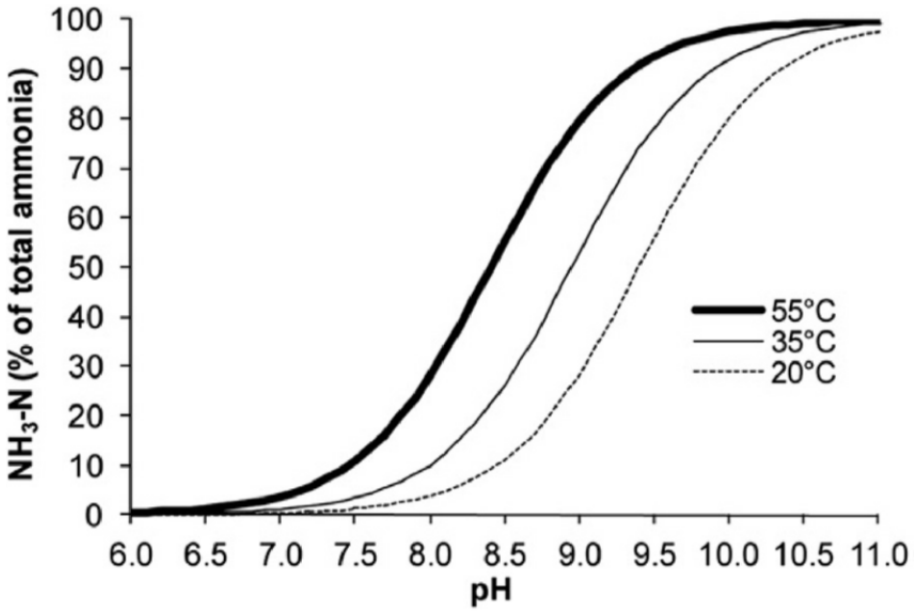

Figure 2. Free ammonia percentage in solution at 20, 35 and 55 °C and varying pH [Adapted from (Fernandes et al., 2012)].

However, up to now, there is no practically feasible solution to overcome ammonia toxicity effect in full-scale biogas reactors. Solutions such as ammonia stripping or dilution of reactor contents with water are economically unattractive or environmentally unfavourable due to the increased waste volume (Nielsen & Angelidaki, 2008). Moreover, another optional valid solution is decreasing of temperature, which leads to the reducing of free ammonia concentrations. However, the metabolic rate of the microorganisms involved is lower at the same time which results in a limitation of the efficiency in this solution (Kayhanian, 1999). Therefore, the solutions used now are either too expensive or time consuming for the full-scale biogas plants. Thus, novel solutions to overcome the problem of ammonia inhibition efficiently and economically are still urgently required.

1.5 Objectives and thesis structure

1.5.1 Specific objectives

In order to alleviate the toxicity effect of high ammonia levels efficiently, some new hypotheses and specific objectives of the study were presented as followed.

Increasing the C/N ratio is a method to counteract ammonia inhibition. This is happening because the microorganisms can use the ammonia nitrogen to produce biomass. Preliminary modelling results from a previous study, have demonstrated that the increase of lipids' concentration in ammonia-rich substrates, could theoretically mitigate the ammonia inhibition problem (Angelidaki et al., 1999). Therefore, the first specific objective was to evaluate the interaction between lipids and high ammonia loading during continuous methane production and the effect of lipids to counteract ammonia inhibition in lab scale reactors. Additionally, for comparison purposes, a soluble carbohydrate (i.e. glucose) was also used as a co-substrate in an identical CSTR reactor (Paper III).

Additionally, the problem of ammonia inhibition during biomethanation process could also be solved by microbiological methods. It has been reported that aceticlastic methanogens are more sensitive to ammonia toxicity effect compared with hydrogenotrophic methanogens (Angelidaki & Ahring, 1993; Koster & Lettinga, 1984). Therefore, it is possible to promote the syntrophic acetate oxidation pathway during biomethanation process for counteracting ammonia inhibition. Thus, the second specific objective was to explore the microbiological mechanisms that dictating the robustness of the hydrogenotrophic methanogens to ammonia toxicity and research on how syntrophic acetate oxidizers and hydrogenotrophic methanogens are influenced by the different operational conditions (ammonia levels) was done (Paper II).

Based on the same idea introduced above (promoting the syntrophic acetate oxidation pathway to alleviate ammonia inhibition), a hypothesis that it is possible to use bioaugmentation with high ammonia tolerant hydrogenotrophic methanogens to solve the problem of ammonia inhibition successfully was presented. Therefore, the third specific objective was to assess the effect of the bioaugmentation with high ammonia tolerant hydrogenotrophic methanogens on fast recovery from ammonia inhibition during biomethanation process (Paper I).

In addition, an innovative method, where hydrogen is injected in the anaerobic reactor and subsequently is converted together with carbon dioxide to methane by hydrogenotrophic methanogens, has been developed for simultaneous biogas production and upgrading (chemoautotrophic biogas upgrading). Thus, such novel biomethanation process could potentially be more tolerant or robust to ammonia toxicity due to the enrichment of hydrogenotrophic methanogens compared to the conventional biomethanation processes, which has never been explored so far. Therefore, the fourth specific objective was to assess the effect of different ammonia levels on this hydrogen assisted biogas upgrading process under different hydrogen partial pressure (0, 0.25, 0.5 and 1 atm) in anaerobic reactors at both mesophilic and thermophilic temperature (Paper IV).

1.5.2 Structure of the thesis

In Chapter 2, the introduction of the method of improving C/N ration to overcome ammonia inhibition was presented and the calculation of microbial biomass generation and ammonium nitrogen fixation when using different substrates was described. In addition, the interaction between lipids (glucose) and high ammonia concentrations during continuous biomethanation process, and the effect of lipids (glucose) to alleviate ammonia toxicity were discussed in this chapter (Paper III).

In Chapter 3, the effects of different ammonia levels on pure strains of syntrophic acetate oxidation bacteria and hydrogenotrophic methanogens were evaluated and the interactions between syntrophic acetate oxidation bacteria and hydrogenotrophic methanogens under different ammonia concentrations were assessed. The main results of Paper II were shown in this chapter.

In Chapter 4, the state of the art of bioaugmentation was discussed. Moreover, the effect of the bioaugmentation with high ammonia tolerant hydrogenotrophic methanogens on fast recovery from ammonia inhibition during biomethanation process was also evaluated. The main results of Paper I were shown in this chapter.

In Chapter 5, the introduction of chemoautotrophic biogas upgrading and the enrichment of hydrogenotrophic methanogens by adding hydrogen were presented. In addition, the effect of different ammonia levels on hydrogen assisted biogas upgrading process in anaerobic reactors at both mesophilic and thermophilic temperature was evaluated and the main results of Paper IV were shown in this chapter. Conclusions and future perspectives follow.

2 Co-digestion with lipids to alleviate ammonia inhibition

Increasing the C/N ratio in anaerobic reactors has been considered as a practical solution for ammonia inhibition (Rajagopal et al., 2013). During anaerobic digestion process, low C/N ratio could cause ammonia accumulation and then inhibit the growth of the anaerobic microorganisms (Resch et al., 2011). On the contrary, organic overloading and the accumulation of VFA concentrations could be caused when the C/N ratio is too high (Nagao et al., 2012). Therefore, the ammonia toxicity problem in continuous reactors could be theoretically relieved by improving the C/N ratio (i.e. co-digestion with a substrate that contains high carbon) to an optimal level (between 16/1 and 25/1) (Shanmugam & Horan, 2009). In addition, considering there is no additional equipment needed, it is a practical and economically attractive solution for alleviating ammonia inhibition in full-scale biogas reactors.

2.1 Anaerobic co-digestion for improving C/N ratio

There have been many researches focused on improving C/N ratio with co-digestion. For example, a study about comparing the effect of different C/N ratios between 3.2 and 30 on alleviating ammonia toxicity was made by Shanmugam and Horan (2009) using blended leather fleshing waste as substrate. The results showed that when the C/N ratio reached 15/1, more accumulative methane production was achieved. In another study, animal by-products (mixture of swine blood, rumen content and wastewater with 6.61 g NH_4^+-N L^{-1}) and starch were co-digested which increased the C/N ratio from 9.7 to 18.3, resulting in 55% higher methane production compared to the reactor without co-digestion (Resch et al., 2011). Similarly, Hejnfelt and Angelidaki (2009) observed that in mesophilic condition, an increase in methane production (40%) was achieved when pig manure was co-digested with pork by-products (5%) compared to the reactors digested pig manure alone. Moreover, in Kafle et al. (2012)'s study, the performances of anaerobic reactors with different C/N ratios (2.3, 7.15 and 12.16) were compared. Two different ratios of swine manure and waste silage (VS: waste silage/swine manure 33/67 and 67/33 %) were used to improve the C/N ratios and the results showed that 19% and 40% higher of methane productions were obtained, respectively.

When C/N ratio was increased, ammonia levels could be reduced with the growing of anaerobic microorganisms (producing protein). However, it only happens when the methanogens in the reactors are still active (Rajagopal et al., 2013). Therefore, it is a valid solution for optimizing the C/N ratio through co-digestion with carbon-rich wastes to relieve the problem of ammonia inhibition during anaerobic digestion (Kayhanian, 1999). However, there are still contrary results in some previous studies. Resch et al. (2011) reported that the methane yield decreased 78.7% when glycerine was mixed in the substrate to increase the C/N ratio at an ammonia concentration of 6.61 g NH_4^+-N L^{-1}. Therefore, the problem of which kind of substrates should be used for co-digestion to mitigate ammonia toxicity on anaerobic digestion remains.

2.2 Microbial biomass generation and ammonium nitrogen fixation by different substrates

When glucose, glycerol trioleate (GTO) and gelatin are individually degraded during anaerobic digestion, different amounts of microbial biomasses per substrate mass are generated. Thus, the microorganisms will uptake different amounts of ammonium nitrogen in order to create the different amounts of cell biomasses in the reactors. Therefore, glucose, GTO and gelatin were used as model carbohydrate, lipid and protein, respectively and the corresponding degradation process were introduced as followed.

2.2.1 Carbohydrates-Glucose

For the degradation of soluble carbohydrates, there are three main steps. Firstly, acetate ($C_2H_4O_2$), propionate ($C_3H_6O_2$) and butyrate ($C_4H_8O_2$) are produced from the degradation (Eq. (4)) (Angelidaki et al., 1993). Secondly, acetate and hydrogen (H_2) are generated from the consuming of propionate and butyrate (Eq. (5) (6)) (Fedorovich et al., 2003). Thirdly, methane is produced by hydrogen-using methanogens (Eq. (7) (8)) and aceticlastic methanogens (Eq. (9)) (Hill, 1982). The whole process is based on the following equations.

$$(C_6H_{10}O_5)_s + 0.1115\ NH_3 \rightarrow 0.1115\ C_5H_7NO_2 + 0.744\ C_2H_4O_2 + 0.5\ C_3H_6O_2 + 0.4409\ C_4H_8O_2 + 0.6909\ CO_2 + 0.0254\ H_2O \quad (4)$$

$$C_3H_6O_2 + 1.764\ H_2O + 0.0458\ NH_3 \rightarrow 0.0458\ C_5H_7NO_2 + 0.9345\ C_2H_4O_2 + 2.804\ H_2 + 0.902\ CO_2 \quad (5)$$

$C_4H_8O_2$ +1.7818 H_2O + 0.0544 NH_3 + 0.0544 CO_2 → 0.0544 $C_5H_7NO_2$ + 1.8909 $C_2H_4O_2$ + 1.8909 H_2 (6)

2.804 H_2 + 0.01618 NH_3 + 0.7413 CO_2 → 0.001618 $C_5H_7NO_2$ + 0.6604CH_4 + 1.45 H_2O (7)

1.8909 H_2 + 0.0109 NH_3 + 0.4999 CO_2 → 0.0109 $C_5H_7NO_2$ + 0.4452CH_4 + 0.978 H_2O (8)

$C_2H_4O_2$ + 0.022 NH_3 → 0.022 $C_5H_7NO_2$ + 0.945 CH_4 + 0.066 H_2O + 0.945 CO_2 (9)

2.2.2 Lipids-GTO

The degradation of GTO is a process of breaking down of lipids to release oleate and glycerol. Then the glycerol is converted into propionate and biomass (Eq. (10)) (Angelidaki et al., 1999). Thus, the oleate is an intermediate, which will be consumed by LCFA degrading acetogenic bacteria to generate acetate and H_2 (Eq. (11)). The consuming of acetate was described above (Eq. (9)) (Considering the consuming of propionate is an integral part of the hydrolysis of GTO, it is no kinetically need to include propionate in the model (Schauder & Schink, 1989)). The whole process is based on the following equations (Angelidaki et al., 1999).

$C_{57}H_{104}O_6$ + 1.90695 H_2O + 0.04071 NH_3 + 0.0291 CO_2 → 0.04071 $C_5H_7NO_2$ + 0.941843 $C_3H_6O_2$ + 3 $C_{18}H_{34}O_2$ (10)

$C_{18}H_{34}O_2$ + 7.7401 H_2O + 4.0834 CO_2 + 0.2537 NH_3 → 0.2537 $C_5H_7NO_2$ + 8.6998 $C_2H_4O_2$ + 3.4139 CH_4 (11)

2.2.3 Proteins-Gelatin

For the degradation of gelatin, different VFA (acetate, propionate, butyrate, and valerate) are produced (Eq. (12)). Thus, the consuming of acetate, propionate, and butyrate was based on the previous model (equation (9), (5) and (6), respectively). Furthermore, equation (13) is shown for the consuming of valerate. Therefore, the whole process is based on the following equations (Angelidaki et al., 1999; Angelidaki et al., 1993).

$CH_{2.03}O_{0.6}N_{0.3}S_{0.001}$ + 0.3006 H_2O → 0.017013 $C_5H_7NO_2$+ 0.29742 $C_2H_4O_2$ + 0.02904 $C_3H_6O_2$ + 0.022826 $C_4H_8O_2$ + 0.013202 $C_5H_{10}O_2$ + 0.07527 CO_2 + 0.28298 NH_3 + 0.001 H_2S (12)

$C_5H_{10}O_2$ + 0.0653 NH_3 + 0.5543 CO_2 + 0.8045 H_2O → 0.0653 $C_5H_7NO_2$ + 0.8912 $C_2H_4O_2$ + 0.02904 $C_3H_6O_2$ + 0.4454 CH_4 (13)

Overall, there is more energy content in lipids compared with proteins and carbohydrates. Thus, 0.0043 g and 0.1312 g more NH_4^+-N are captured (resulting in relieving the ammonia inhibition), when 1 g GTO is consumed as substrate compared to glucose and gelatine as substrates, respectively (table 2).

In addition, the initial modelling results from a previous study have also demonstrated that the increasing of lipids concentrations in high ammonia content substrate for feeding anaerobic reactors, could theoretically mitigate the ammonia inhibition problem (Angelidaki et al., 1999). Hence, the co-digestion of high ammonia content substrates with lipids, is a possible practical method to decrease the ammonia concentration in a continuous reactor and then to alleviate the ammonia inhibition, which is the hypothesis of the current study. However, the interaction between lipids and high ammonia concentrations during continuous biomethanation process, and the effect of lipids to alleviate ammonia toxicity, are still unclear. Additionally, Park and Li (2012) observed that microorganisms' activity could be inhibited and the crucial groups on the cell membranes could be disoriented by the presence of long chain fatty acids (LCFA).

Table 2. Microbial biomass generation and ammonium nitrogen fixation by different substrate

Substrate	Ammonium nitrogen fixation (g NH_3-N g^{-1} substrate)	Maximum microbial biomass yield (g biomass g^{-1} substrate)
$C_{57}H_{104}O_6$ (GTO)	0.0230	0.18
$C_6H_{10}O_5$ (Glucose)	0.0187	0.15
$CH_{2.03}O_{0.6}N_{0.3}S_{0.001}$ (Gelatin)	-0.1082	0.11

Therefore, the effect of co-digestion of cattle manure with lipid (i.e. GTO) or soluble carbohydrate (i.e. glucose) on relieving the ammonia inhibition under high ammonia levels (5 g NH_4^+-N·L^{-1}) in anaerobic continuous stirred tank reactors (CSTR) was assessed in the current study. The same operating conditions were applied for both reactors using GTO (R_{GTO}) or glucose (R_{GLU}) as co-substrates. In addition, a reactor fed with only dairy manure was used as control reactor (R_{CTL}). Furthermore, the specific methanogenic activity (SMA) of all the reactors was tested under 5 g NH_4^+-N·L^{-1} to assess the combining effect of high ammonia levels and GTO or glucose on the growth of methanogenic populations in the CSTR reactors.

In this experiment, at the beginning the substrate for all the three reactors was only manure and the organic loading rate (OLR) was 2.2 g $VS \cdot L^{-1} \cdot d^{-1}$. After that, the OLR of R_{GTO} and R_{GLU} was increased stepwise to 3 and 4 g $VS \cdot L^{-1} \cdot d^{-1}$ (by adding GTO and glucose into the substrate, respectively), while the OLR of R_{CTL} remained the same (2.2 g $VS \cdot L^{-1} \cdot d^{-1}$). When all the reactors reached a steady-state (Phase I), the ammonia levels in the substrate for all three reactors was increased stepwise to 4 (Phase II) and 5 g NH_4^+-$N \cdot L^{-1}$ (Phase III).

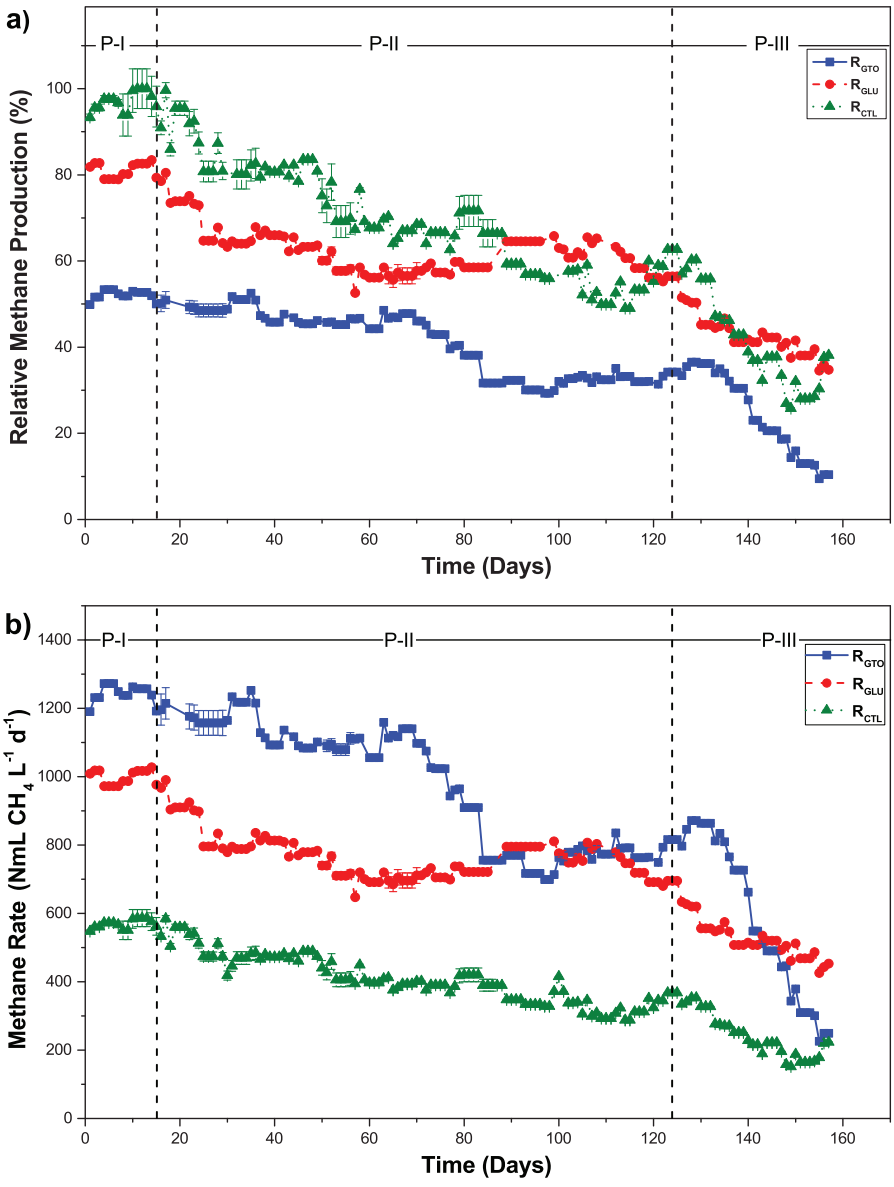

Figure 3. a) Relative methane production and b) methane production rate of the three CSTR reactors. Phase-I, ammonia concentration: 2.1 g NH_4^+-$N \cdot L^{-1}$; phase-II, ammonia concentration: 4 g NH_4^+-$N \cdot L^{-1}$; and phase-III, ammonia concentration: 5 g NH_4^+-$N \cdot L^{-1}$ [Adapted from Paper III].

The results of the current study (Paper III) indicated that after increasing ammonia levels (4 and 5 g NH_4^+-N·L^{-1}), instead of relieving ammonia toxicity effect, as it was hypothesized, a totally inhibition of anaerobic digestion process was caused by co-digestion with GTO. However, the reactor co-digestion with glucose showed a significantly more robust performance to high ammonia levels compared with co-digestion with GTO under 4 and 5 g NH_4^+-N·L^{-1} (the same OLR). The only explanation for this result was the different nature of GTO and glucose. It seems that an obvious synergistic inhibitory effect was caused by the LCFA cooperated with high ammonia concentrations which deteriorated the inhibition of the anaerobic digestion process. In a previous study, it was reported by Lü et al. (2013) that high VFA and ammonia concentrations would generate a synergistic inhibitory effect in anaerobic digestion. However, the "ammonia-LCFA synergetic inhibitory effect" caused by co-digestion with GTO seems to have a more toxicity effect compared with the "ammonia-VFA synergetic inhibitory effect" during anaerobic digestion in the current study due to the better performance of co-digestion with glucose. The reason for this might be that VFA is not a primary inhibitor, and is just a production of an inhibited process.

During anaerobic digestion process, inhibition caused by ammonia toxicity effect and LCFA individually were both reported before (reviewed by (Chen et al., 2014; Yenigün & Demirel, 2013)). However, about the "ammonia-LCFA synergetic inhibitory effect", there is only few studies involved in this topic (e.g. (Astals et al., 2014)). Moreover, the mechanisms of the inhibition, the interaction of the LCFA and high ammonia levels and biochemical parameters of the "ammonia-LCFA synergetic inhibitory effect" are still unclear and further studies are needed.

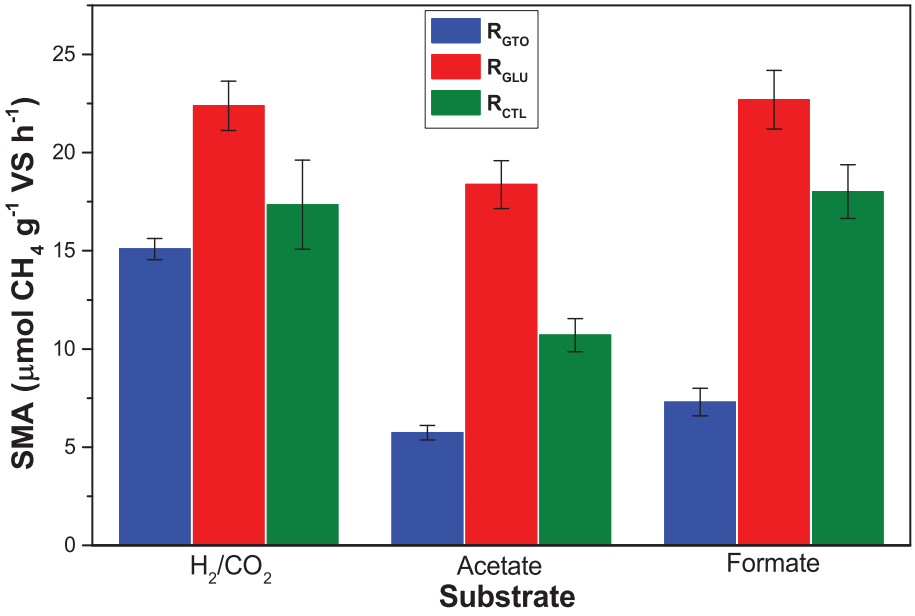

Figure 4. Specific methanogenic activity (SMA) measured in different reactors.

Hydrogenotrophic methanogens in all three reactors had the highest SMA compared with aceticlastic methanogens and formate-utilization methanogens. The results of SMA test in the current study were in agreement with many previous studies which demonstrated that aceticlastic methanogens are more sensitive to high ammonia levels compared with hydrogenotrophic methanogens (Yenigün & Demirel, 2013).

Furthermore, the results of SMA indicated that compared to aceticlastic and formate utilizing methanogens, the hydrogenotrophic methanogens were more tolerant to ammonia-LCFA synergistic inhibitory effect. Moreover, the tolerance to other toxic conditions has been demonstrated in some previous studies for the hydrogenotrophic methanogens (Lopez et al., 2013; Symsaris et al., 2015).

Overall, the hypothesis that the co-digestion of cattle manure with lipids could theoretically alleviate the ammonia inhibition was not supported by the results. However, an "ammonia-LCFA synergetic inhibitory effect" was observed in the reactor using GTO as co-substrate, which caused a deterioration of the inhibition in anaerobic digestion process. On contrary, the reactor co-digested with glucose was more tolerant to high ammonia levels and had a better performance compared with the reactor co-digested with GTO.

3 Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate oxidizing bacteria

3.1 Ammonia effect on hydrogenotrophic methanogens

Microbiological methods could also be used as a solution for ammonia inhibition. It is possible to promote the microorganisms robust to ammonia during biomethanation process for counteracting ammonia toxicity effect. For consuming acetate to produce methane, the two different pathways (the aceticlastic pathway and the SAO pathway) have been introduced above (Chapter 1.2.4). It has been reported (literature reviewed by (Yenigün & Demirel, 2013)) that the methanogens mediating these two pathways have different sensitivities to ammonia. In detail, there are many previous studies demonstrating that aceticlastic methanogens are more vulnerable to ammonia toxicity effect compared to hydrogenotrophic methanogens (Angelidaki & Ahring, 1993; Koster & Lettinga, 1984). However, some researchers also reported several contrary results. Specifically, in Angelidaki and Ahring (1994)'s study, 3.5 g NH_4^+-N L^{-1} was defined as a threshold of ammonia inhibition for hydrogenotrophic methanogens. Additionally, when the ammonia concentration increased to 7 g NH_4^+-N L^{-1}, the growth rate of the methanogens decreased 50% at thermophilic condition (pH 7.9-8.0). In another study, under mesophilic conditions, when ammonia concentrations were 4.2 and 5.6 g NH_4^+-N L^{-1}, there was no ammonia inhibition observed for the hydrogenotrophic methanogen (*Methanobacterium* strain G2R) (Sprott & Patel, 1986). Therefore, the ammonia tolerance of the hydrogenotrophic methanogens still needs further studied.

3.2 Ammonia effect on syntrophic acetate oxidizing bacteria

Zinder and Koch (1984) first observed the SAO pathway in a thermophilic reactor and then another study reported that the same pathway was found in sludges at lower temperature under high ammonia concentration (Schnürer et al., 1996). Moreover, with the increasing temperature, the energy of overall reaction is enhanced (Schink & Stams, 2013). So far, six SAOB have been described and isolated; three mesophilic: *Syntrophaceticus schinkii*

(Westerholm et al., 2010), *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011) and *Clostridium ultunense* (Schnürer et al., 1996) and three thermophilic: *Thermacetogenium phaeum* (Hattori et al., 2000), *Thermotoga lettingae* (Balk et al., 2002) and strain AOR (Lee & Zinder, 1988). In detail, there are some previous studies indicating that some pure strains of SAOB (*C. ultunense*, *T. acetatoxydans* and *S. schinkii*) could overcome the high ammonia toxicity effect (8.4-14 g NH_4^+-N L^{-1}) (Schnürer et al., 1996; Westerholm et al., 2010; Westerholm et al., 2011). In another study, Kato et al. (2014) observed that when the ammonia concentration was increased to 2.8 g NH_4^+-N L^{-1}, the growing of *T. phaeum* suffered an inhibition. However, the information of *T. lettingae*'s ability to overcome high ammonia levels is still lacking (Sun et al., 2014).

3.3 Ammonia effect on the syntrophic cultivation

SAOB cannot oxidise acetate all by itself. Hydrogenotrophic methanogens (i.e. *Methanomicrobiales* spp., *Methanococcales* spp., *Methanocellales* spp., *Methanobacteriales* spp. and *Methanopyrales* spp.) which can consume hydrogen and carbon dioxide are crucial to keep a low partial hydrogen pressure environment so SAOB can keep using acetate in the first step of SAO pathway (Angelidaki et al., 2011). Therefore, the collaboration of SAOB and hydrogenotrophic methanogens is vital in the SAO pathway. Additionally, the cooperation of SAOB and hydrogenotrophic methanogens is based on interspecies hydrogen transfer (Stams et al., 2006). Moreover, there are also several studies assessing the ammonia toxicity on the co-cultures of SAOB and hydrogenotrophic methanogens. It has been reported that at 1.4 g NH_4^+-N L^{-1}, the growth of the syntrophic cultivation of *T. phaeum* and *Methanothermobacter thermautotrophicus* was not affected. Furthermore, under 2.8 g NH_4^+-N L^{-1} the methane production decreased about 50% and deteriorated at 7 g NH_4^+-N L^{-1} (Kato et al., 2014).

However, so far, the tolerant ability to different ammonia levels of the syntrophic cultivation of SAOB and hydrogenotrophic methanogens are still unclear. Additionally, the interactions between SAOB and hydrogenotrophic methanogens under different ammonia concentrations and the role that each one of them plays during the SAO pathway need to be studied further.

Therefore, in the current study (paper II) the effects of different ammonia levels on pure strains of SAOB and hydrogenotrophic methanogens were evaluated. Furthermore, the effect of different ammonia levels on the co-cultivation of SAOB and hydrogenotrophic methanogens was also assessed.

In this experiment, four hydrogenotrophic methanogens (mesophilic*: Methanobacterium congolense C* DSM No. 7095 and *Methanoculleus bourgensis MS2* DSM No. 3045; thermophilic: *Methanothermobacter thermautotrophicus Z-245* DSM No. 3720 and *Methanoculleus thermophilus* UCLA DSM No. 2624.) and two SAOB (Mesophilic: *Tepidanaerobacter acetatoxydans* Re1^{T} DSM No. 21804; thermophilic: *Thermacetogenium phaeum strain PB* DSM No. 26808) were obtained from DSMZ company (Germany) and were used for evaluating their tolerance to different ammonia levels. All hydrogenotrophic methanogens and SAOB were cultivated under four different ammonia and free ammonia concentrations. In addition, syntrophic cultivation experiment (SAOB and hydrogenotrophic methanogens cultivated together) under the same four different ammonia concentrations was also tested in the current study.

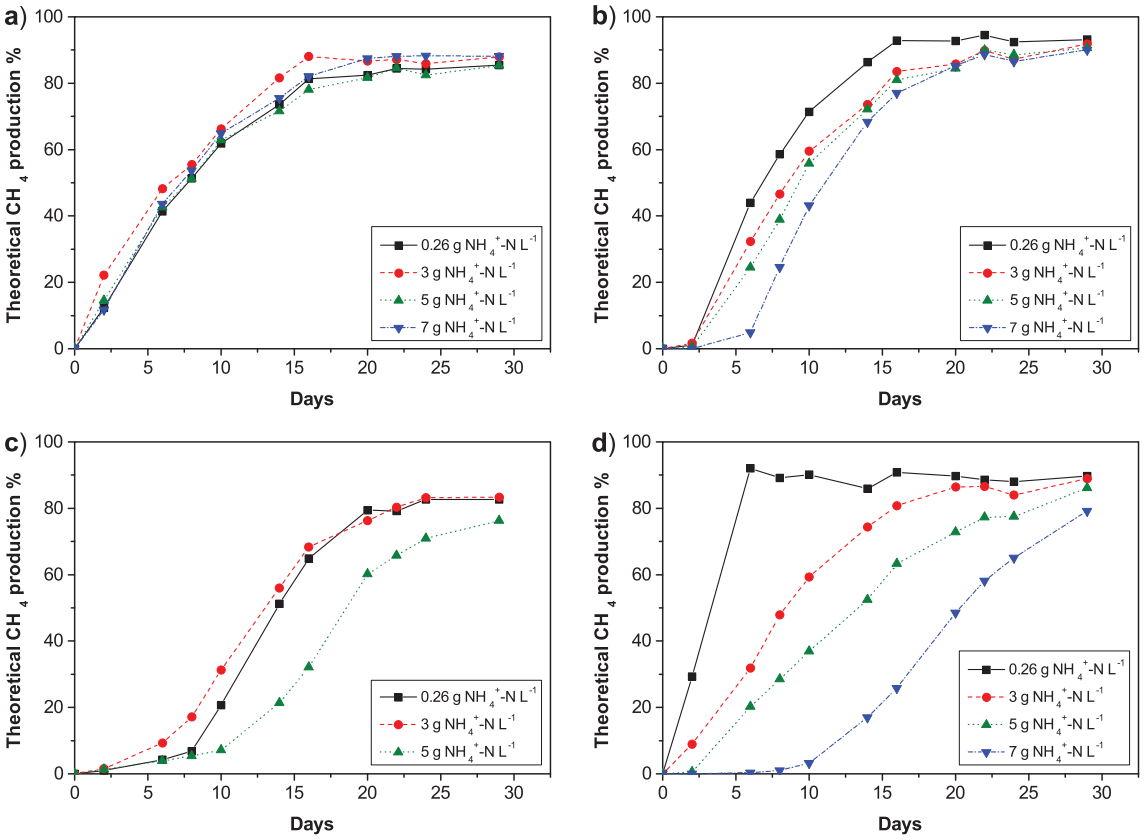

Figure 5. Accumulative methane production under different ammonia levels of a) *M. bourgensis,* b) *M. thermophiles,* c) *M. congolense* and d) *M. Thermautotrophicus* [Adapted from Paper II].

The results from Paper II showed that as the ammonia concentrations were increased, the methane production of *M. congolense* and *M. thermautotrophicus* had a significant ($p < 0.05$) reducing, while there was no significant ($p >$

0.05) effect on the methane production of *M. bourgensis* and *M. thermophiles*. Specifically, the serious ammonia inhibition on the *M. thermautotrophicus* was due to the higher free ammonia concentrations in thermophilic condition compared to the mesophilic hydrogenotrophic methanogens. Interestingly, the result of *M. thermophiles* demonstrated that there also exist thermophilic hydrogenotrophic methanogens, which are tolerant to high ammonia and free ammonia levels, even more robust compared to mesophilic hydrogenotrophic methanogen (*M. congolense*).

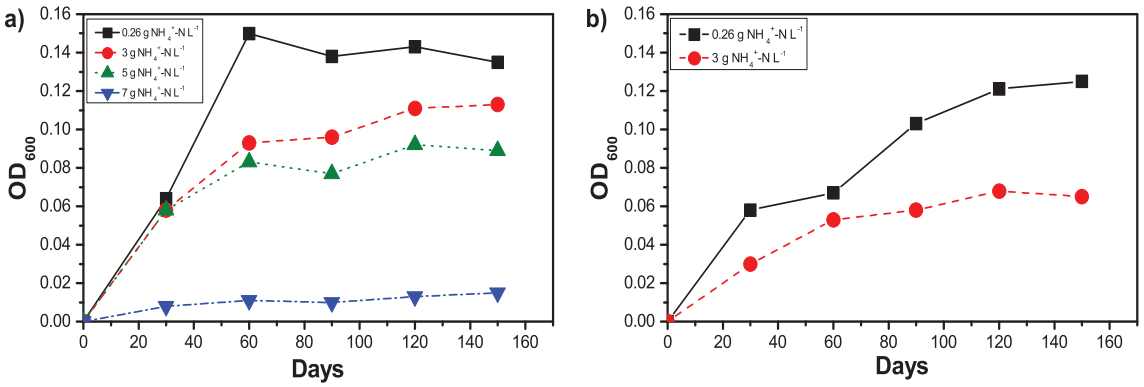

Figure 6. The OD_{600} under different ammonia levels of a) T. acetatoxydans and b) T. phaeum. [Adapted from Paper II].

Alongside the increase of ammonia levels, a significant ($p < 0.05$) inhibition was observed during the cultivation of *T. acetatoxydans* and *T. Phaeum*. The ammonia inhibition clearly indicated that the tested hydrogenotrophic methanogens (except *M. congolense*) were more tolerant to ammonia inhibition compared to the two SAOB tested in the current study. However, it has been reported that methanogens are more vulnerable to high ammonia concentrations compared to SAOB (Fotidis et al., 2013b), which was contradictory to the results that we obtained. Therefore, it seems that there are some hydrogenotrophic methanogens equally or more tolerant to high ammonia concentrations compared to some SAOB.

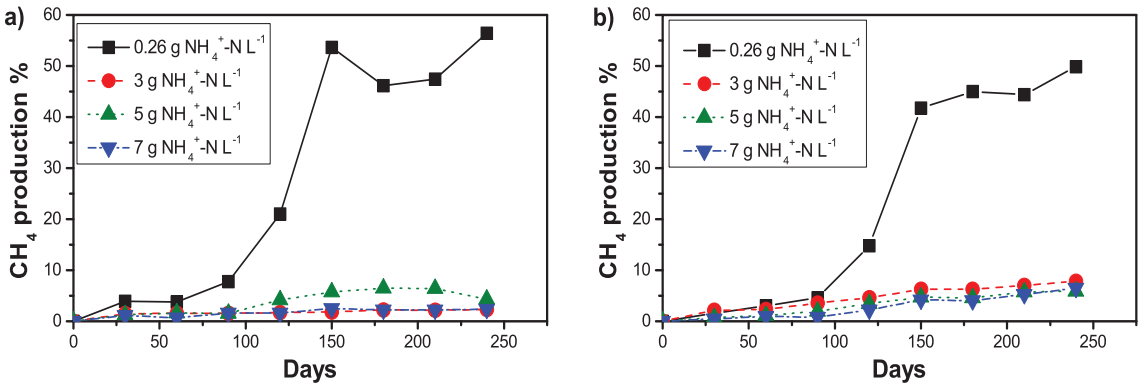

Figure 7. The methane production under different ammonia levels of syntrophically cultivated microbes: a) *M. thermophiles* + *T. phaeum* and b) *M. Thermautotrophicus* + *T. phaeum*. [Adapted from Paper II].

For the thermophilic syntrophic cultivation, when ammonia concentration increased, the methane production was significantly ($p < 0.05$) reduced. Furthermore, more severe ammonia inhibition occurred in the mesophilic syntrophic cultivation: the methane production was under detection limit when ammonia levels reached 3 g NH_4^+-N L^{-1}. In addition, an interesting result was observed that under the ammonia levels of 5 and 7 g NH_4^+-N L^{-1}, methane production was detected from the thermophilic syntrophic cultivation while the OD_{600} of *T. phaeum* was under detection limits. Therefore, it seems that the resistance of the SAO consortium to high ammonia levels could be enhanced when it was syntrophically cultivated with hydrogenotrophic methanogens. The reason might be that hydrogen was consumed by the hydrogenotrophic methanogens, which improved the growth of SAOB. Thus, it indicated that under high ammonia concentrations, hydrogenotrophic methanogens seem to be the critical factor in the SAO pathway.

As conclusions, the results of paper II showed that there exists some hydrogenotrophic methanogens, which were equally, or more resistant to ammonia inhibition compared to SAOB. In addition, compared to mesophilic hydrogenotrophic methanogen (*M. congolense*), thermophilic hydrogenotrophic methanogen tested (*M. thermophiles*) in the current study was more robust to high ammonia concentrations. Moreover, for SAOB, the resistance to ammonia toxicity could be improved by syntrophic cultivation with hydrogenotrophic methanogens, which indicated that at high ammonia levels, hydrogenotrophic methanogens seem to play the essential roles in the SAO pathway. Overall, the main results of paper II demonstrated that it is difficult to generalise for the ammonia inhibition effect on the microorganisms involved in anaerobic digestion process.

4 Bioaugmentation as a solution to overcome ammonia inhibition

4.1 Background of bioaugmentation

Bioaugmentation is a technology in which specific microorganisms are introduced to a biological system resulting in increasing the activity of selected microorganisms (Deflaun & Steffan, 2002; Rittmann & Whiteman, 1994). For its effect of improving the performance of reactors, it has been widely applied both in aerobic and anaerobic conditions.

4.1.1 Bioaugmentation applied in aerobic and anaerobic process

In aerobic process, the growth of nitrifying bacteria could be impacted by cold temperature, excess biomass washout, inappropriate pH and different kinds of inhibition caused by different toxic materials and after applying bioaugmentation the population of nitrifying bacteria was increased (Abeysinghe et al., 2002; Head & Oleszkiewicz, 2005; Rittmann & Whiteman, 1994; Satoh et al., 2003). Moreover, there are also other problems in aerobic process, which could be solved by using bioaugmentation. In Singer et al. (2005)'s study, bioaugmentation could be applied in the bioremediation of polluted soil. Additionally, Van Limbergen et al. (1998) reported that the degradation of recalcitrant compounds could also be enhanced by using bioaugmentation.

During anaerobic processes, there are many studies reported that the degradation of different kinds of substrate could be improved after bioaugmentation. Angelidaki and Ahring (2000) observed that the degradation of hemicellulose was increased and 30% higher of methane production rate was achieved by using bioaugmentation. In another study, Nielsen et al. (2007a) reported that a improving of 93% in the methane production yield was obtained by the bioaugmentation with *Caldicellusiruptor* in a thermophilic anaerobic reactor feeding with cattle manure. Moreover, some researches about the enhanced degradation of other substrates (such as tetrachloroethylene, pentachlorophenol, phenol and fat) after bioaugmentation were also reported (Charest et al., 1999; Cirne et al., 2006; Guiot et al., 2002; Guiot et al., 2000; Hörber et al., 1998; Levesque & Tartakovsky, 1999). Additionally, other applications of bioaugmentation have been validated. In Stephenson and Stephenson (1992)'s study, the reactors suffering organic overload were recovered by bioaugmentation. Costa et al. (2012) reported that the start-up period of the

anaerobic digestion process was shortened when *Caldicellulosiruptor saccharolyticum* was used for bioaugmentation.

4.1.2 Problems exist in bioaugmentation

However, several problems also exist in the technology of bioaugmentation. There are some reasons that caused the failure of bioaugmentation (El Fantroussi & Agathos, 2005; Goldstein et al., 1985; Lange et al., 1988; Martin Jr & Zall, 1985; Qasim & Stinehelfer, 1982; Wilderer et al., 1991):

- When an unsuitable microorganism (grow in high temperatures) was introduced into reactors with low temperatures during anaerobic digestion process, the added microorganisms may not survive.
- The adaptation problems of the introduced microorganism may occur, which resulted in a failure of the bioaugmentation.
- The competition between the introduced microorganism and the indigenous microorganisms could also cause a failure of the bioaugmentation.
- The beneficial effect could be reduced if the volume of the bioaugmentation was not enough.
- The recovery of methane production yield could only be kept for a limited time. In addition, the added microorganisms could be washed out from the continuous reactors after bioaugmentation (Nielsen et al., 2007a).

Additionally, in some previous studies, it has been reported that the specific microorganisms could be acclimatised and obtained the beneficial property all by themselves. Therefore, there was no need to use bioaugmentation (Chen et al., 2008; Yenigün & Demirel, 2013).

4.2 Bioaugmentation of high ammonia tolerant methanogens

As it introduced above (Chapter 1.2.4), aceticlastic and SAO pathways are two different pathways for consuming acetate to produce methane. For the tolerance to high ammonia levels, the results of many studies demonstrated that aceticlastic methanogens are more sensitive to ammonia toxicity compared to hydrogenotrophic methanogens (Angelidaki & Ahring, 1993; Koster & Lettinga, 1984).

Therefore, the ammonia tolerant SAO pathway could be enhanced by bioaugmentation to alleviate ammonia inhibition in anaerobic digestion process. However, the results of Westerholm et al. (2012) and Fotidis et al. (2013a)'s

studies showed that the bioaugmentation of high ammonia tolerant syntrophic methanogenic consortia (syntrophic combination of SAOB and a hydrogenotrophic methanogen) was very difficult (if possible) in continuous reactors.

In SAO pathway, keeping hydrogen partial pressure low is necessary. The methane producing from propionate and other VFA would be enhanced in low hydrogen concentration (<50 mM) (McCarty & Smith, 1986). Furthermore, the results of Fotidis et al. (2013a)'s study also indicated that in the high ammonia tolerant syntrophic combination (SAOB and hydrogenotrophic methanogens), hydrogenotrophic methanogens are the rate limiting microorganisms. Thus, hydrogenotrophic methanogens rather than the SAOB, play the key role in the anaerobic digestion process under high ammonia concentrations and determine the growth rate of the syntrophic combination in continuous reactors.

Therefore, for fast recovery from ammonia inhibition during anaerobic digestion process, the bioaugmentation of high ammonia tolerant methanogenic archaea could be a new practical solution. Thus, this new practical bioaugmentation method to mitigate ammonia toxicity in a CSTR reactor (R_{MC}) under mesophilic conditions and at suboptimal state (which was caused by high ammonia levels), was tested in the current study (paper I). In a previous study, it was reported that *Methanoculleus bourgensis* $MS2^T$ was an ammonia tolerant methanogen which could grow under 5 g NH_4^+-N L^{-1} (Ollivier et al., 1986). Therefore, *M. bourgensis* was selected as the rapid growing hydrogenotrophic methanogen for bioaugmentation under high ammonia concentrations. Additionally, another CSTR reactor without bioaugmentation ($R_{Control}$) was used to compare and assess the effect of alleviating ammonia inhibition under the same operational conditions.

This experiment was divided into three distinct experimental phases:

- Phase-I (days 1−12), one hydraulic retention time (HRT) after ammonia concentration of the substrate was increased to 5 g NH_4^+-N·L^{-1}, an ammonia induced "inhibited steady-state" was established for both reactors in this phase. The "steady-state" was assumed, as the variation of the methane yield was less than 10% for at least ten consecutive days.
- Phase-II (days 13−15), the bioaugmentation process of the *M. bourgensis* in the R_{MC} reactor was performed in two distinct steps (days 13 and 15, respectively).

- Phase-III (days 16−57), the reactors were operated continuously and the ammonia concentration in the substrate was kept at 5 g NH_4^+-N·L^{-1}.

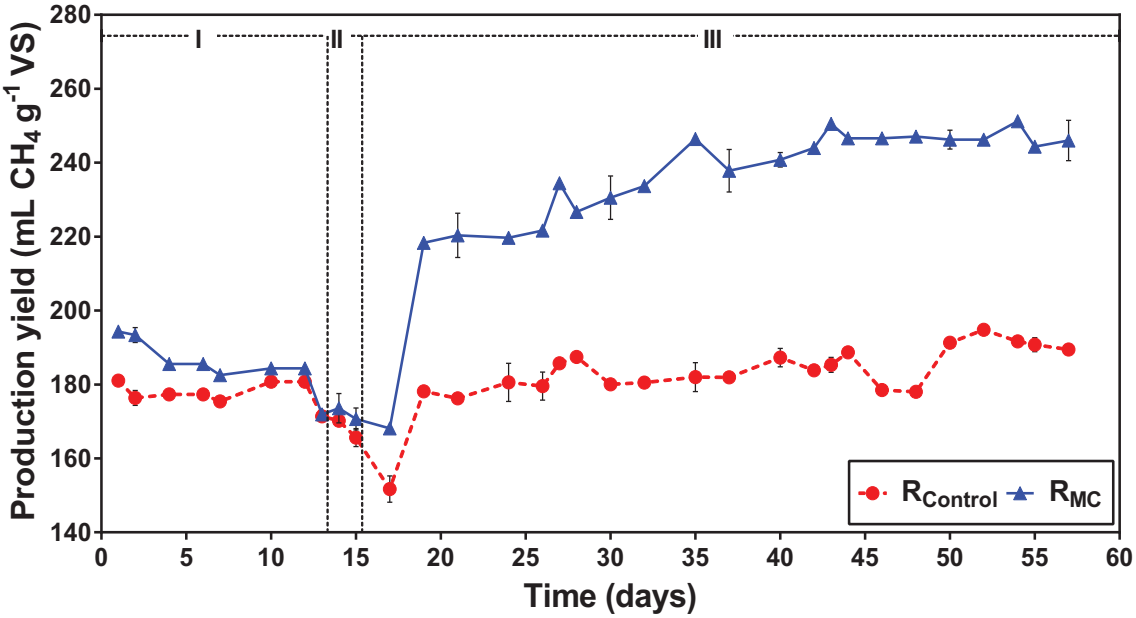

Figure 8. Methane production yield of the two CSTR reactors. Phase-I, before bioaugmentation and abiotic augmentation; phase-II, bioaugmentation and abiotic augmentation; and phase-III, after bioaugmentation and abiotic augmentation. Error bars denote standard deviation from the mean of triplicate measurements (n = 3). [Adapted from Paper I]

The results of the current study (paper I) showed that there was a significant increasing ($p < 0.05$) of methane production yield detected in the R_{MC} reactor. Moreover, the increased methane production yield was kept more than one HRT during the experiment (the average of methane production yield was 31.8% higher compared with the $R_{Control}$ reactor). The significant methane production recovery from ammonia inhibition by bioaugmentation in anaerobic continuous reactors was never reported in previous studies.

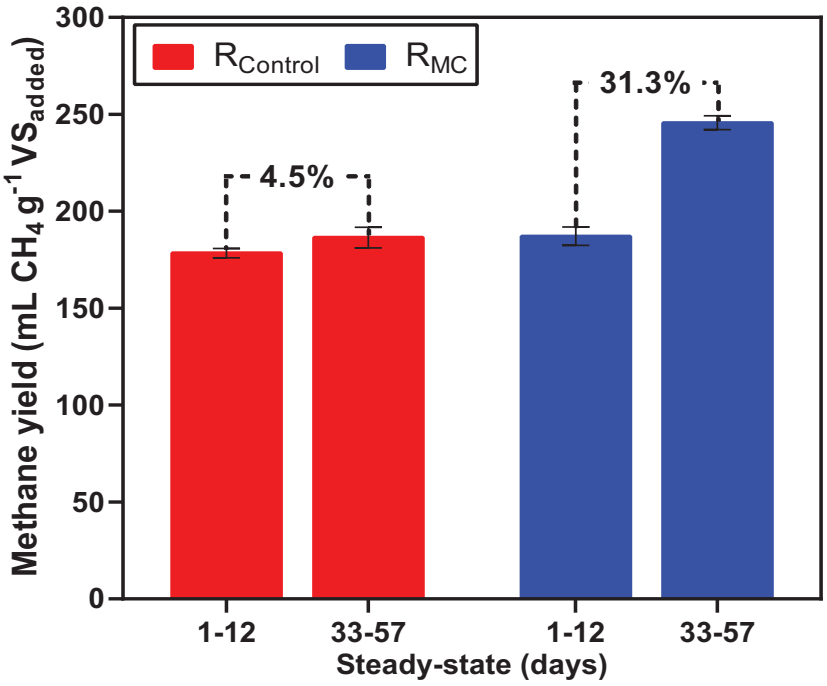

Figure 9. Average methane production yield comparison under different steady-states before and after abiotic augmentation and bioaugmentation for reactors $R_{Control}$ and R_{MC}, respectively. [Adapted from Paper I]

In the R_{MC} reactor, the amount of accumulated VFA decreased significantly ($p < 0.05$) compared to the $R_{Control}$ reactor during the final steady-state (days 33−57) at 5 g NH_4^+-N·L^{-1} (Figure 10), which was in agreement with the results of methane production yield. In detail, the total VFA accumulation concentrations of the R_{MC} reactor after bioaugmentation, was kept stable and within the threshold for a healthy anaerobic digestion process of dairy slurry in CSTR reactors (Fang et al., 2011).

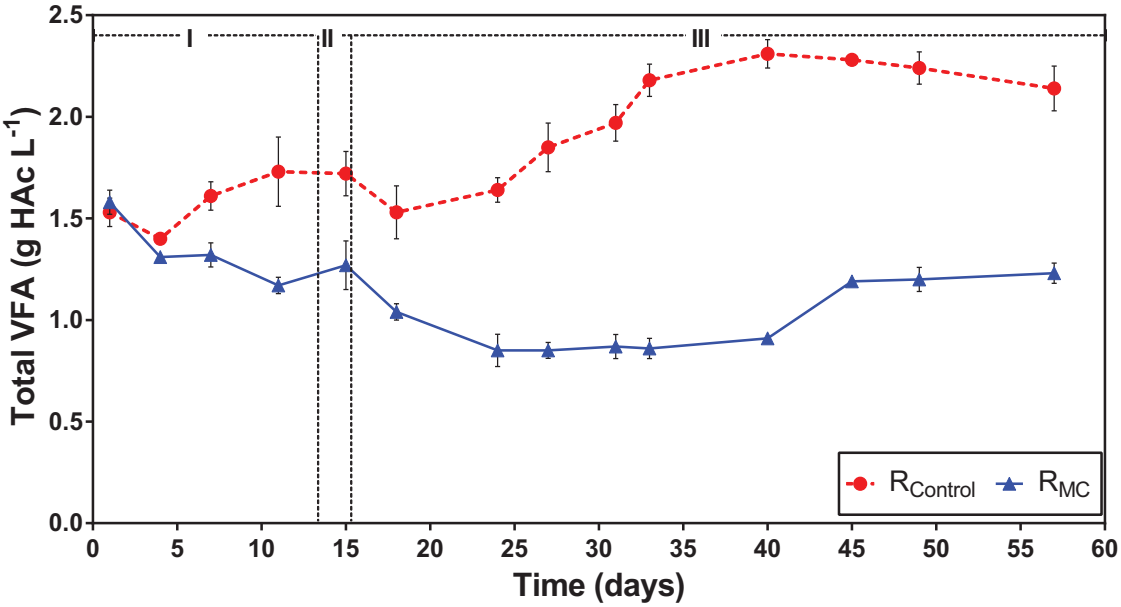

Figure 10. Total VFA accumulation in CSTR reactors. Phase-I, before bioaugmentation and abiotic augmentation; phase-II, bioaugmentation and abiotic augmentation; and phase-III, after bioaugmentation and abiotic augmentation. Error bars denote standard deviation from the mean of triplicate measurements (n = 3). [Adapted from Paper I]

Therefore, this new solution to counteract ammonia inhibition was proven to be more practical and effective compared with other methods applied today in continuous reactors (Nielsen & Angelidaki, 2008). Furthermore, bioaugmentation with an ammonia tolerant methanogen to alleviate ammonia toxicity could be applied for improving the efficiency of biomethanation process in full-scale continuous reactors.

In addition, the "critical biomass", which was described as the minimum volume of specific microorganisms' biomass that was bioaugmented in continuous reactors to avoid being washed out, is the essential for the success of bioaugmentation experiment. However, it is still unclear if there existed less biomass amount of introduced microorganism for bioaugmentation, which can solve the ammonia inhibition in continuous reactors. Thus, more studies are needed to work on this problem.

5 Ammonia effect on hydrogen assisted biogas production and upgrading process

Chemoautotrophic biogas upgrading, where hydrogen is injected in the anaerobic reactor and subsequently been converted together with carbon dioxide to methane by hydrogenotrophic methanogens, has been developed for simultaneous biogas production and upgrading. Thus, such novel biomethanation process could potentially be more tolerant or robust to ammonia toxicity due to the enrichment of hydrogenotrophic methanogens compared to the conventional biomethanation processes. Therefore, it could be applied as a practical solution for overcoming ammonia inhibition.

5.1 Chemoautotrophic biogas upgrading

The chemoautotrophic biogas upgrading is dependent on the activity of hydrogenotrophic methanogens. During this process, hydrogen is used as electron donor and carbon dioxide is electron acceptor and carbon source, as shown in Equation (3) (Chapter 1.2.4) (Strevett et al., 1995).

Upgrading biogas by using hydrogen to generate methane from carbon dioxide has been widely applied (Ju et al., 2008; Kim et al., 2013). Moreover, even syngas of carbon dioxide, carbon monoxide and hydrogen could be converted to methane by some methanogens, which could use carbon monoxide to generate methane and carbon dioxide as the following equation showed:

$$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2 \qquad (14)$$

Many methanogens (*Methanosaeta* spp., *Methanobacterium* spp., *Methanosarcina* spp., *Methanoculleus* spp., *Methanococcus* spp., *Methanospirillum* spp. and *Methanothermobacter* spp.) have been reported that keep being presence in anaerobic reactors for upgrading biogas by adding external hydrogen (Kim et al., 2013; Luo & Angelidaki, 2013; Luo et al., 2012; Strevett et al., 1995; Wang et al., 2013). Moreover, in both mesophilic and thermophilic conditions, the suitable range of pH levels is from 6.5 to 8 for these methanogens, which are applied for biogas upgrading. Furthermore, part of the hydrogen sulfide in the biogas could also be consumed by methanogens to generate biomass.

Additionally, it has been reported that the temperature could affect the conversion process for producing methane (Strevett et al., 1995). In detail, the mesophilic methanogens could convert carbon dioxide more completely

compared with thermophilic methanogens. Furthermore, lower growth rates could be obtained by thermophilic methanogens, which would improve the generation of methane from carbon dioxide instead of producing microbial biomass.

The stoichiometric ratios of hydrogen and carbon dioxide are mostly selected as 4:1 in biogas upgrading process at lab scope (both in mesophilic and thermophilic conditions) by consuming hydrogen in anaerobic reactors (Kim et al., 2013). However, a problem affects the conversion rate from carbon dioxide to methane. Specifically, according to Henry's law, the aqueous solubility for hydrogen is low which would cause the limitation of the transfer rate of hydrogen from gas to water. Therefore, the low hydrogen concentration in aqueous phase led to a low conversion rate to methane (Strevett et al., 1995). Thus, a high retention time for gas is needed to obtain a satisfactory methane concentration (more than 90%) for biogas upgrading, but meanwhile, the methane production is kept low. On the other hand, when the methane production was increased during a short retention time, the methane concentration was decreased which is not acceptable for biogas upgrading.

There are several previous studies that assessed the effect of hydrogen addition in the anaerobic reactors directly for biogas upgrading (Luo & Angelidaki, 2013; Luo et al., 2012). In Luo and Angelidaki (2013)'s study, there was no additional anaerobic reactors needed in the experiment. Furthermore, a crucial coenzyme's activity (F_{420}), which was related with aceticlastic and hydrogenotrophic methanogens, was enhanced and another enzyme's activity (acetate kinase) which was essential to the producing of acetate from VFA was not reduced. In addition, a higher specific ATP content was achieved which resulted in an improvement of microorganism's activity by introducing hydrogen in anaerobic reactors compared with the reactor without additional hydrogen.

Additionally, there are many advantages for applying chemoautotrophic biogas upgrading in biogas plants: (1) by using hydrogen and carbon dioxide to produce methane in only one anaerobic reactor, biogas upgrading can be achieved which is economic attractive for improving the quality of biogas to natural gas. Thus, in this way the biogas could be also used as vehicle fuel (Deng & Hägg, 2010). (2) Akansu et al. (2004) has observed that the combustion capability of biogas could be enhanced by the mixture with the hydrogen (5–30% in volume) unconverted when used as fuel. (3) Since methane has a higher volumetric energy density and boiling point, it will cost much less to

store methane compared with hydrogen (Balat, 2008). (4) In biogas plants, the basic facilities that already existed could be used for biogas upgrading.

However, there are also some problems caused by injecting hydrogen into anaerobic reactors. For example, an accumulation of VFA (propionate and butyrate) concentration was created by the raising of the hydrogen partial pressure in the anaerobic reactors (Fukuzaki et al., 1990; Siriwongrungson et al., 2007).

5.2 The effect of ammonia on hydrogen assisted biogas production and upgrading process

In fact, the enrichment of hydrogenotrophic methanogenic cultures in anaerobic biogas reactors by introducing hydrogen has been studied before. Specifically, In Luo and Angelidaki (2012)'s study, a hydrogen partial pressure of 0.8 atm was obtained in anaerobic reactors by adding hydrogen. After the cultivation of two months, the hydrogenotrophic methanogenic activities increased 188 mL CH_4/ (g VSS h) under mesophilic condition and 310 mL CH_4/ (g VSS h) under thermophilic condition compared with the beginning of the enrichment which indicated that hydrogenotrophic methanogens were selectively enriched successfully by introducing hydrogen.

Thus, in the current study (paper IV), it would be obvious to hypothesize that this process could potentially be more resistant to high ammonia levels due to the enrichment of hydrogenotrophic methanogens compared to the conventional anaerobic digestion processes. However, so far, it is still unclear about the effect of different ammonia levels on hydrogen assisted biogas upgrading process. Therefore, the effect of different ammonia concentrations on this innovative anaerobic digestion process in anaerobic reactors at both mesophilic and thermophilic conditions was evaluated in the current study.

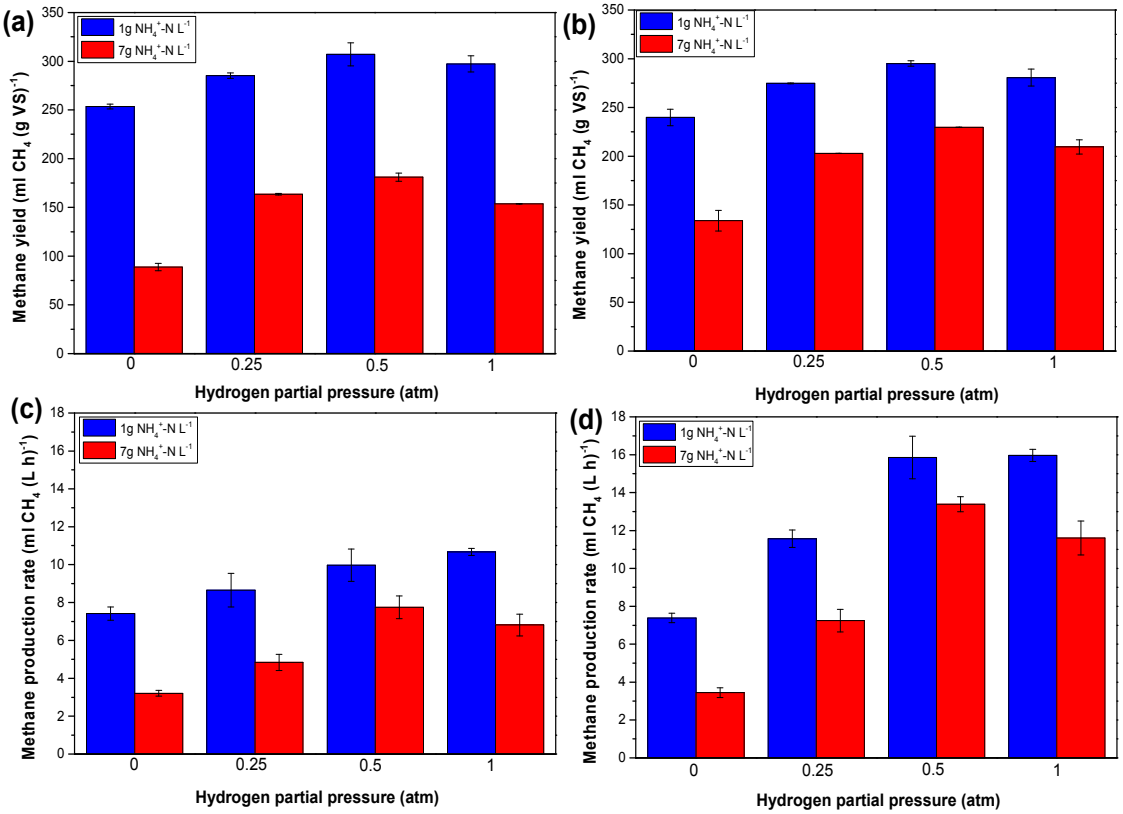

Figure 11. Methane yield as a function of hydrogen partial pressure: (a) mesophilic condition (b) thermophilic condition. Methane production rate as a function of hydrogen partial pressure: (c) mesophilic condition (d) thermophilic condition. [Adapted from Paper IV]

Both the results of the methane yield and methane production rate (Paper IV) demonstrated that the hydrogen assisted biogas production and upgrading processes can still function at high ammonia concentrations, though ammonia inhibition occurred. Under high ammonia concentrations, the optimal hydrogen initial partial pressure for methane production was 0.5 atm. The results also indicated that the hydrogen assisted biogas upgrading process was more robust to high ammonia levels compared to the conventional anaerobic digestion processes. Moreover, an interesting finding was that thermophilic batch reactors had higher methane yield compared to mesophilic reactors under high ammonia levels (5 and 7 g NH_4^+-N L^{-1}) at 0.5 atm. This result differed from some previous studies (Chen et al., 2008; Fotidis et al., 2013b), which indicated that mesophilic methanogens are more tolerant to high ammonia levels compared to the thermophilic methanogens due to the lower free ammonia levels. However, it was also (Wang et al., 2015) reported that some hydrogenotrophic mesophilic methanogens was more vulnerable to high am-

monia and free ammonia levels compared with thermophilic methanogens, which was in accordance with the result of the current study.

Overall, the results of the current study indicated that the hydrogen assisted biogas production and upgrading processes were inhibited by high ammonia levels. Nevertheless, the hydrogen assisted biogas upgrading process was still more robust to the increasing ammonia concentrations compared to the conventional anaerobic digestion processes. Under all the different ammonia concentrations tested in the current study, the optimal hydrogen partial pressure in batch reactors was 0.5 atm. Furthermore, at 0.5 atm of hydrogen partial pressure, the thermophilic methanogens seemed to be more robust to high ammonia concentrations (5 and 7 g NH_4^+-N L^{-1}) compared to mesophilic methanogens.

6 Conclusions

This thesis was focused on the optimization of biomethanation process under high ammonia levels. Some new hypotheses to overcome ammonia inhibition were presented and evaluated in the current study. Overall, the main conclusions of this thesis are summarised as follows:

- At 5 g NH_4^+-N·L^{-1}, relative methane production of R_{GTO} and R_{GLU}, was 10.5% and 41% compared to the expected uninhibited production, respectively. At the same time control reactor (R_{CTL}), only fed with manure, reached 32.7% compared to the uninhibited basis production. Therefore, using lipids as a co-digestion substrate could not alleviate the ammonia toxicity effect on anaerobic digestion process. On contrary, an "ammonia-LCFA synergetic inhibitory effect" was observed when using GTO as co-substrate, which caused a deterioration of the inhibition. On the other hand, the reactor co-digested with glucose was more tolerant to high ammonia levels compared with the reactor co-digested with GTO.
- Compared to aceticlastic and formate utilizing methanogens, the hydrogenotrophic methanogens were more tolerant to ammonia-LCFA synergistic inhibitory effect.
- A 31.3% increase in methane production yield was observed in the CSTR reactor, at steady-state, after bioaugmentation. Therefore, the new solution of bioaugmentation with an ammonia tolerant methanogen to alleviate ammonia toxicity effect is more practical and effective compared with other method applied today in continuous reactors (Nielsen & Angelidaki, 2008). Furthermore, it could be applied for improving the efficiency of biomethanation process in full-scale continuous reactors.
- There existed some hydrogenotrophic methanogens (79.1% of the theoretical methane production) which were equally, or more resistant to ammonia inhibition compared to SAOB (11.1% of the theoretical methane production).
- Thermophilic hydrogenotrophic methanogen tested in the current study (*M. thermophiles*) was more robust to high ammonia concentrations compared to the mesophilic hydrogenotrophic methanogen (*M. congolense*).
- At high ammonia levels, hydrogenotrophic methanogens seem to play the essential roles in the SAO pathway.

- When the initial hydrogen partial pressure was 0.5 atm, the methane yield at high ammonia load (7 g NH_4^+-N L^{-1}) was 41.0% and 22.3% lower than at low ammonia load (1 g NH_4^+-N L^{-1}) in mesophilic and thermophilic condition, respectively. For the reactors without adding hydrogen, the methane yield decreased 65.0% (mesophilic) and 44.2% (thermophilic) when ammonia level increased to 7 g NH_4^+-N L^{-1}. Therefore, the hydrogen assisted biogas production and upgrading processes were inhibited by high ammonia levels. Nevertheless, the hydrogen assisted biogas upgrading process was still more robust to the increasing ammonia concentrations compared to the conventional anaerobic digestion processes.
- Under all the different ammonia concentrations tested in the current study, the optimal hydrogen partial pressure in batch reactors was 0.5 atm.
- At 0.5 atm of hydrogen partial pressure, the thermophilic methanogens seemed to be more robust to high ammonia concentrations (5 and 7 g NH_4^+-N L^{-1}) compared to mesophilic methanogens.

7 Future perspectives

In this thesis, several different novel methods to alleviate ammonia toxicity effect during anaerobic digestion process were presented and discussed. However, there still remain some problems in the current study and further works are also needed.

- In the study of bioaugmentation, it is still unclear if there existed less biomass amount of introduced microorganism for bioaugmentation, which can alleviate the ammonia toxicity in continuous reactors. Thus, further studies are needed to work on this problem. Moreover, future work on evaluating the effect of fast recovery from ammonia inhibition by bioaugmentation with high ammonia tolerant methanogenic archaea under thermophilic conditions or under higher ammonia concentrations (> 5 g NH_4^+-N L^{-1}) is also needed.
- In the research of using lipids as co-substrate to alleviate the ammonia inhibition, an "ammonia-LCFA synergetic inhibitory effect" was created. There is only few studies focused on this topic (e.g. (Astals et al., 2014)). Therefore, the mechanisms of this inhibition, the interaction of the LCFA and high ammonia levels and biochemical parameters of the "ammonia-LCFA synergetic inhibitory effect" are still unclear and further studies are needed.
- The hydrogen assisted biogas upgrading process in anaerobic batch reactors was proven more tolerant to high ammonia levels compared to the conventional anaerobic digestion processes. However, further works about the effect of hydrogen assisted biogas upgrading process on improving ammonia tolerance in continuous reactors under high ammonia levels are still needed in the future.

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9 Papers

I Fotidis, I.A., Wang, H., Fiedel, N.R., Luo, G., Karakashev, D.B., Angelidaki, I. 2014. Bioaugmentation as a solution to increase methane production from an ammonia-rich substrate. *Environmental Science & Technology*, 48(13), 7669-7676. DOI: 10.1021/es5017075.

II Wang, H., Fotidis, I.A., Angelidaki, I. 2015. Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate-oxidizing bacteria. *FEMS Microbiology Ecology*, 91(11), fiv130. DOI: http://dx.doi.org/10.1093/femsec/fiv130.

III Wang, H., Fotidis, I.A., Angelidaki, I. 2016. Ammonia - LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process. *Bioresource Technology*. Accepted.

IV Wang, H., Zhang, Y., Angelidaki, I. 2016. Ammonia effect on hydrogen assisted biogas production and upgrading process. Submitted.

In this online version of the thesis, papers **I-IV** are not included but can be obtained from electronic article databases, e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
Water Resources Engineering, Urban Water Engineering,
Residual Resource Engineering and Environmental Chemistry & Microbiology.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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